Polyadenylation of mRNA: Minimal Substrates and a Requirement for the 2' Hydroxyl of the U in AAUAAA

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mRNA-specific polyadenylation can be assayed *in vitro* by using synthetic RNAs that end at or near the natural cleavage site. This reaction requires the highly conserved sequence AAUAAA. At least two distinct nuclear components, an AAUAAA specificity factor and poly(A) polymerase, are required to catalyze the reaction. In this study, we identified structural features of the RNA substrate that are critical for mRNA-specific polyadenylation. We found that a substrate that contained only 11 nucleotides, of which the first six were AAUAAA, underwent AAUAAA-specific polyadenylation. This is the shortest substrate we have used that supports polyadenylation: removal of a single nucleotide from either end of this RNA abolished the reaction. Although AAUAAA appeared to be the only strict sequence requirement for polyadenylation, the number of nucleotides between AAUAAA and the 3' end was critical. Substrates with seven or fewer nucleotides beyond AAUAAA received poly(A) with decreased efficiency yet still bound efficiently to specificity factor. We infer that on these shortened substrates, poly(A) polymerase cannot simultaneously contact the specificity factor bound to AAUAAA and the 3' end of the RNA. By incorporating 2'-deoxyuridine into the U of AAUAAA, we demonstrated that the 2' hydroxyl of the U in AAUAAA was required for the binding of specificity factor to the substrate and hence for poly(A) addition. This finding may indicate that at least one of the factors involved in the interaction with AAUAAA is a protein.

The mature 3' termini of most eucaryotic mRNAs are formed by cleavage of the mRNA precursor and addition of poly(A) to the newly formed end (reviewed in references 3, 10, 11, and 18). Two sequences are required for efficient cleavage: the highly conserved AAUAAA sequence, commonly located 15 to 25 nucleotides upstream of the poly(A) site, and a less conserved sequence downstream of the poly(A) site. AAUAAA is also required for polyadenylation of synthetic RNAs that end at the poly(A) site, so-called precleaved RNAs. This requirement is observed both *in vitro*, in a HeLa cell nuclear extract (e.g., reference 28) and *in vivo*, after injection into frog oocyte nuclei (6a).

Cleavage and polyadenylation occur in a specific processing complex, as demonstrated by sedimentation (16, 23), RNase resistance (8, 9, 23), oligonucleotide and RNase H analysis (23, 29), and nondenaturing gel electrophoresis (9, 22, 30, 31). The latter assay reveals the existence of a distinct postcleavage complex, obtained if polyadenylation is blocked after cleavage (30). Synthetic RNAs that end at the poly(A) site form the postcleavage complex *de novo* (29, 30).

At least three separable factors are required for cleavage: a specificity factor, a cleavage factor, and a poly(A) polymerase (4, 5, 7, 12, 24, 25). Although none are yet pure, it is clear that only the specificity factor and polymerase fractions are required for AAUAAA-specific poly(A) addition to precleaved RNAs (5, 7). Candidates for the factor that recognizes AAUAAA have been identified, though none have been demonstrated directly to play a role in the

reaction. These include U11 small nuclear ribonucleoprotein (snRNP) (4) and a 64- or 68-kilodalton protein that can be cross-linked by UV light to RNAs containing AAUAAA (1, 15, 26). The enzyme that adds poly(A) is a classical poly(A) polymerase (2, 5, 24). It becomes selective for AAUAAA-containing RNAs by interacting with specificity factor (2, 4, 12, 24).

The use of precleaved RNAs makes possible the analysis of polyadenylation alone, uncoupled from cleavage. The polyadenylation observed in these uncoupled reactions is very similar if not identical to that which occurs after cleavage, as judged by several criteria. First, both cleavage and polyadenylation are dependent on AAUAAA (29). Second, AAUAAA mutants with different severities affect the two reactions comparably (M. Sheets, manuscript in preparation). Third, substrates for both reactions form very similar complexes with processing factors (29, 30). Similarly, uncleaved precursors, cleaved RNAs, and precleaved RNAs all can be UV cross-linked to a 64- or 68-kilodalton protein (1, 15, 26). Fourth, poly(A) addition and cleavage apparently require common specificity factors and poly(A) polymerase. This was initially deduced from competition experiments (29) and has since been demonstrated more conclusively by copurification of AAUAAA-specific cleavage and polyadenylation activities (4, 5, 25).

In this report, we examine detailed requirements for polyadenylation *in vitro*, when uncoupled from cleavage. We identify a minimal substrate for polyadenylation and analyze the effect of altering the distance between AAUAAA and the 3' end of an RNA. We investigate the mechanism by which AAUAAA is recognized by determining the effect of perturbing the ribose moiety of the U in AAUAAA.

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MATERIALS AND METHODS

Transcription in vitro. RNAs were prepared by in vitro transcription of synthetic oligonucleotide templates with T7 RNA polymerase (New England BioLabs, Inc.), as described previously (14) except that our T7 promoter oligonucleotide extends from -17 to -1 rather than to +1. Also, some T7 transcriptions were performed by using the buffer for SP6 RNA polymerase (13); the RNA yield is equivalent to that obtained with the buffer of Milligan et al. (14). α -³²P-labeled nucleoside triphosphates were included in transcription reactions to label RNAs. 2'-Deoxyuridine (2'-dU) was incorporated by inclusion of 100 μ M 2'-dUTP (Pharmacia) in the transcription reaction, with no UTP added. All RNAs lacked a cap. Transcripts were purified by electrophoresis through polyacrylamide gels containing 7 M urea.

As described by Milligan et al. (14), the yield of transcripts synthesized by T7 polymerase varies considerably, depending on the first few nucleotides in the transcript. For example, a high yield is obtained with GG or GC as the first two nucleotides, whereas initiating with AAA or AUA results in very low yields.

RNA substrates. All RNAs correspond to the region upstream of the simian virus 40 (SV40) late mRNA poly(A) site (designated +1) or are variants of this sequence. All sequences are given in the 5'-to-3' direction.

(i) **-23/+1 and derivatives.** -23/+1 is a 24-nucleotide RNA, representing the SV40 late sequence from 23 nucleotides 5' to and including the poly(A) site at +1. The sequence of -23/+1 RNA is GCUGCAAUAAACAAGUUAACAACA. Shorter RNAs (-23/-2, -3, -4, -5, -6, -7, -8, -9, and -10) were isolated as premature termination products of a transcription reaction of the full-length -23/+1 template. -23/+1 mutant RNA contains AAUACA rather than AAUAAA.

(ii) **-20/-7 and derivatives.** A DNA template for -20/-7 RNA was used to generate -20/-6, -7, -8, and -9 RNAs. The sequence of -20/-7 RNA is GCAAUAAACAAGUU. -20/-7 single-U RNA was transcribed from a different DNA and contains an altered -20/-7 sequence, with only a single U and a single C residue. The sequence of this RNA is GGAAUAAACAAGAA.

(iii) **-38/+1 single-U RNA.** -38/+1 single-U RNA represents the SV40 late sequence from -36 to +1, with two additional G residues at the 5' end and with all U residues changed to C except for the U in AAUAAA. A total of seven uridines have been changed. Its sequence is GGGCAACCA CCACAAGCCGCAAUAAACAAGCCAACAACA. In spite of the seven mutations, this RNA is polyadenylated as efficiently as the natural sequence in the combined Mono Q and DE-600 fractions (not shown).

(iv) **Minimal substrates and derivatives.** The sequences of these RNAs differ entirely from that of SV40 RNAs. The sequences of these RNAs are AAUAAACCCCA, AAGAAA CCCC, AAUAAACCCC, AUAAACCCCA, and AAUAAACCCA. These RNAs are analogous to -18/-8 and -18/-9 RNAs and contain only 10 or 11 nucleotides.

Characterization of RNAs. To characterize purified transcription products, transcripts were analyzed by digestion with RNase T₁. Labeled RNAs (10 fmol, mixed with 25 μ g of yeast RNA) were digested with RNase T₁ (5 U) at 37°C for 30 min. Digestion products were analyzed by electrophoresis on denaturing polyacrylamide gels next to RNase T₁ digestion fragments of known length. To identify the 3'-terminal oligonucleotide, T₁ digestion products were treated with calf

intestinal phosphatase. All internal oligonucleotides decrease in electrophoretic mobility after treatment with phosphatase, since phosphatase removes the 3' phosphate groups left by RNase T₁. Only the 3'-terminal T₁ oligonucleotide, which already has a 3' hydroxyl group, is unaffected in mobility by phosphatase. We used this lack of effect to identify this oligonucleotide.

-23 RNAs were characterized by their migration on denaturing gels relative to RNase T₁ digestion products of known length and by comparison with -23/-9 RNA. This RNA (GCAAUAAACAAG) was readily identified because unlike all other RNAs in the -23 series, it ends in a G residue. RNase T₁ treatment of this RNA generates CAAUAAACAAG_{OH}; RNase T₁ treatment of all longer RNAs (e.g., -23/-8) generates the same oligonucleotide carrying a 3'-terminal phosphate instead. Thus, phosphatase treatment changes the mobility of this oligonucleotide when it is derived from -23/+1 to -23/-8 but does not affect its mobility when it is derived from -23/-9 RNA. With shorter RNAs (i.e., -23/-10), the 3'-terminal oligonucleotide is increased in mobility and insensitive to phosphatase.

Minimal substrates were identified by their migration on denaturing gels relative to -23 RNAs of known sequence.

RNase T₂ analysis. To analyze the nucleotide composition of RNAs, labeled RNAs (10 fmol, mixed with 12.5 μ g of yeast RNA) were digested with RNase T₂ (1.5 U) at 37°C for approximately 16 h. Digestion products were separated by two-dimensional thin-layer chromatography as described previously (17, 20).

Polyadenylation assays. All polyadenylation reactions were carried out in vitro, using chromatographic fractions derived from a HeLa nuclear extract. Nuclear extract was prepared as described previously (29). Two fractions were combined for the assays: a Mono Q fraction [containing the poly(A) polymerase] and a DE-600 fraction (which confers AAU AAA specificity to the reaction). These two fractions have been described previously (2) and are analogous to crude polymerase and specificity factor fractions described by Christofori and Keller (4, 5). Reaction mixtures contained 3 μ l of the Mono Q fraction (in modified buffer D [29]), 3 μ l of the DE-600 fraction (in modified buffer D), 0.24 mM MgCl₂, 20 mM phosphocreatine, 0.1 mM ATP, and 5 to 20 fmol of RNA in a total volume of 12.5 μ l. Incubations were performed at 30°C for 0 to 30 min. RNAs were purified by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation and then analyzed by electrophoresis on denaturing polyacrylamide gels.

Each of the short substrates used in this study (-23/+1, -20/-7, -38/+1 single-U, and minimal RNAs) are polyadenylated more efficiently in the combined Mono Q and DE-600 fractions than in the unfractionated nuclear extract. The biochemical basis of this difference has not yet been determined but may involve inhibitors that are present in crude extract but missing in the more purified fractions and that interfere with polyadenylation of short RNAs.

Analysis of processing complexes. AAUAAA-specific polyadenylation complexes were analyzed on nondenaturing polyacrylamide gels (30). RNA (5 to 20 fmol) was incubated with 3 to 6 μ l of DE-600 in the buffer conditions described above (total volume of 12.5 μ l). 3'-dATP (1 mM) was included in the reaction mixture. After incubation at 25°C for 2 min, 4 μ l was removed and added to 1 μ l of heparin to give a final concentration of 0.5 mg/ml. This sample was loaded directly onto a nondenaturing, 4% polyacrylamide gel as described previously (30).

Quantitation. Autoradiograms were scanned by a laser

microdensitometer. For discussion of the quantitation of complex formation, see the legend to Fig. 3. For quantitation of the effects of substitution with 2'-dU, see the legend to Fig. 4.

RESULTS

RNA substrates and polyadenylation assays. RNAs were transcribed by T7 RNA polymerase from synthetic oligonucleotide templates. All RNAs correspond to or are variants of the region upstream of the poly(A) site of SV40 virion protein mRNAs except for the minimal substrates analyzed in Fig. 1. The natural polyadenylation site is designated +1. Thus, a -23/+1 RNA contains 24 nucleotides, ending at the natural polyadenylation site. AAUAAA lies between -13 and -18.

Polyadenylation assays were performed by using crude chromatography fractions derived from a HeLa nuclear extract. For AAUAAA-specific polyadenylation, two fractions were combined: a fraction containing an AAUAAA specificity factor and another containing the poly(A) polymerase (see Materials and Methods).

An 11-nucleotide RNA is a minimal substrate. To define a minimal substrate for the polyadenylation reaction, we generated short oligoribonucleotides containing the essential AAUAAA polyadenylation signal. The complete sequences of these RNAs are provided in Fig. 1A, and the results obtained by using them as substrates are presented in Fig. 1B and C. The sequence AAUAAACCCCA was selected because CCCC does not occur in any natural mRNA immediately downstream of AAUAAA.

AAUAAACCCCA was polyadenylated in vitro (Fig. 1B, lanes 1 and 2; Fig. 1C, lanes 1 and 2). The identity of the polyadenylated material was confirmed by its retention on oligo(dT)-cellulose (Fig. 1D, lanes 1 and 2). This reaction was AAUAAA specific: AAGAAACCCCA, containing a single-point mutation in AAUAAA, was not detectably polyadenylated (Fig. 1B, lanes 3 and 4).

AAUAAACCCCA is a minimal polyadenylation substrate, since RNAs that lacked either the first nucleotide (AUAACCCCA; Fig. 1C, lanes 3 and 4) or the last nucleotide (AAUAAACCCC; Fig. 1C, lanes 5 and 6) were no longer polyadenylated. The failure of AAUAAACCCC as a substrate was not due to the terminal cytidine, since AAUAAACCCA also was inert (Fig. 1C, lanes 7 and 8).

Polyadenylation efficiency depends on the distance between AAUAAA and the 3' end. The 3' end of AAUAAACCCCA lies eight nucleotides upstream of the natural polyadenylation site generated by cleavage of SV40 late pre-mRNA (+1 [20]). RNAs that end at that natural site, such as -23/+1 or -141/+1 RNAs, are polyadenylated more efficiently than AAUAAACCCCA (not shown). To determine whether the difference in efficiency was due to the difference in the number of nucleotides between AAUAAA and the 3' end of these RNAs, we prepared substrates that differed systematically in the length of this region. The longest RNA was -23/+1. We tested nine other RNAs, from -23/-2 to -23/-10. Each RNA is shorter than the preceding one by a single nucleotide at the 3' end (Fig. 2A), as was confirmed by RNase T₁ analysis (see Materials and Methods). The ability of these RNAs to support polyadenylation is presented in Fig. 2B.

RNAs with termini at -2, -3, -4, or -5 received poly(A) as efficiently as did RNA that ends at +1, the natural poly(A) site (Fig. 2B, lanes 1 to 10). Between -6 and -8 (lanes 11 to 16), polyadenylation gradually decreased until by -9 and

FIG. 1. A minimal polyadenylation substrate. (A) Sequences of RNA substrates used in the reactions shown in panels B to D. The lengths of these RNAs in nucleotides (nt) and their ability to support polyadenylation are also indicated. (B) AAUAAA-specific polyadenylation of an 11-nucleotide RNA. RNAs were incubated in the Mono Q fraction [containing poly(A) polymerase] plus the DE-600 fraction (containing AAUAAA specificity factor) at 30°C for 0 or 30 min, as indicated above each lane. Lanes: 1 and 2, AAUAAACCCCA; 3 and 4, AAGAAACCCCA. Positions of the starting substrates and the polyadenylated products of the reaction are indicated to the left. In this and all subsequent figures, a black box is used to indicate the substrate. (C) Demonstration that AAUAAACCCCA is a minimal substrate. RNAs were incubated as for panel B. Lanes: 1 and 2, AAUAAACCCCA; 3 and 4, AUAACCCCA; 5 and 6, AAUAAACCCC; 7 and 8, AAUAAACCCA. These very short RNAs were degraded quite rapidly in the fractions, but polyadenylated RNA appeared to be more stable (see legend to Fig. 2C). (D) Demonstration that poly(A) is added to AAUAAACCCCA. AAUAAACCCCA was incubated as for panel B for 30 min, and the polyadenylated and nonpolyadenylated products were separated by oligo(dT)-cellulose chromatography. Lanes: 1, RNA that failed to bind to the column (A⁻); 2, RNA that was retained by and eluted from the column (A⁺).

-10 it was no longer detectable (lanes 17 to 20). Quantitation of these data is presented in Fig. 2C. For comparison, we include data obtained with -20 to -6, -7, -8, -9 RNAs and from reactions with AAUAAACCCCA and AAUAAACCCC (-18 RNAs).

The decrease observed between -5 and -9 cannot reflect a requirement for a certain overall length in the oligoribonucleotide. -20/-6 RNA was polyadenylated but -23/-9 was not, yet both of these RNAs are 15 nucleotides in length. Thus, we conclude that the distance between AAUAAA and the 3' end affects polyadenylation efficiency and that the reaction is optimal with eight or more nucleotides 3' to AAUAAA.

There appear to be no strict sequence requirements for the nucleotides between AAUAAA and the 3' end. AAUAAACCCCA is not found in any natural mRNA yet is a competent substrate (Fig. 1). Similarly, the RNAs in Fig. 2 differ in their precise sequences and in the identity of the nucleotide to

FIG. 2. Dependence of polyadenylation efficiency on the distance between AAUAAA and the 3' end. (A) Sequence of -23/+1 RNA. The 3' termini of the transcripts used in panel B are indicated above the sequence. (B) Polyadenylation of -23 RNAs. -23 RNAs were incubated in the Mono Q fraction plus the DE-600 fraction at 30°C for 0 or 30 min, as indicated above each lane. Lanes: 1 and 2, -23/+1; 3 and 4, -23/-2; 5 and 6, -23/-3; 7 and 8, -23/-4; 9 and 10, -23/-5; 11 and 12, -23/-6; 13 and 14, -23/-7; 15 and 16, -23/-8; 17 and 18, -23/-9; 19 and 20, -23/-10. (C) Quantitation of polyadenylation efficiencies. Autoradiograms of polyadenylation reactions with the -23 RNAs described for panel B as well as with -20/-6, -7, -8, and -9, -18/-8, and -18/-9 RNAs (shown in Fig. 1) were scanned with a laser densitometer. Polyadenylation efficiencies (ratio of polyadenylated RNA to total RNA remaining after the incubation) are presented relative to that of -23/+1 as 100%. -9 and -10 RNAs were not detectably polyadenylated. RNAs that were poor substrates for polyadenylation (e.g., -7 and -8 RNAs) were degraded to a greater extent than were efficient substrates. Results obtained with these RNAs may reflect a competition between polyadenylation, which generated relatively stable polyadenylated RNA, and degradation. Hence, the values given for -7 and -8 RNAs may be artificially high. This does not affect the conclusion that reducing the distance between AAUAAA and the 3' end decreases polyadenylation efficiency.

which poly(A) is added yet yield a single systematic result. On the basis of these and other results (see Discussion), we suspect that AAUAAAN₅ (11 nucleotides of RNA, in which AAUAAA are the first 6) is not an unreasonable representation of a minimal substrate for polyadenylation.

Although the only strict sequence requirement appeared to be AAUAAA, sequences other than AAUAAA could influence polyadenylation efficiency (Fig. 2C). For example, AAUAAACCCCA (-18/-8) was polyadenylated more efficiently than either GCAAUAAACAAGU (-20/-8) or GCU GCAAUAAACAAGU (-23/-8). Importantly, however, all three types of RNA clearly exhibited the requirement for a minimal distance between AAUAAA and the 3' end (Fig. 2B). Thus, our main conclusions are that very short oligoribonucleotides support poly(A) addition but that a minimum distance between AAUAAA and the 3' end is required.

3'-Truncated substrates interact with specificity factors but do not receive poly(A). Substrates containing too few nucleotides downstream of AAUAAA might fail to undergo polyadenylation because they interact inefficiently with either poly(A) polymerase or specificity factor. To distinguish between these possibilities, we examined the interaction between specificity factors and AAUAAA by nondenaturing gel electrophoresis. Labeled RNA was incubated with the specificity factor fraction for 2 min. Heparin was then added to a sample of the reaction, and this sample was loaded directly onto a nondenaturing gel. Interaction between AAUAAA and specificity factors was inferred from the formation of complexes that required AAUAAA and had reduced electrophoretic mobility relative to naked RNA. These same criteria have been used previously with longer RNAs to identify polyadenylation-specific complexes (e.g., references 21, 22, 30, and 31).

We analyzed three RNAs that differ dramatically in polyadenylation efficiency: -23/-5, -23/-7, and -23/-9. As polyadenylation substrates, -23/-5 was fully active, -23/-9 was inert, and -23/-7 was intermediate in activity (Fig. 2B). Despite these extreme differences in polyadenylation activity, these RNAs interacted with specificity factors with comparable efficiency (Fig. 3A, lanes 1 to 3). The complexes observed were AAUAAA specific: -23/+1 RNA containing AAUACA formed no detectable complex (lane 4).

Complex formation and polyadenylation are compared directly in Fig. 3B. The data represent the average of three independent experiments. This analysis confirms that poly(A) addition, not formation of AAUAAA-specific complexes, is prevented by shortening the distance between AAUAAA and the 3' end of the substrate. This is seen most strikingly with -23/-9 RNA. This substrate was not detectably polyadenylated yet formed a complex approximately 60% as efficiently as did -23/-5, which was polyadenylated with maximum efficiency. Similar results were obtained with other substrates. For example, AAUAAACCCCA and AAUAAACCCC formed specific complexes with similar efficiencies (Fig. 3A, lanes 5 and 6; Fig. 3B), but only AAUAAACCCCA was detectably polyadenylated (Fig. 3B and 1C).

The 2'-OH group of the U in AAUAAA is required for efficient polyadenylation. Previous work has shown that AAUAAA is required for polyadenylation (e.g., references 21 and 28), and the experiments described above suggest that it also is virtually sufficient. We sought to examine the requirement for AAUAAA at higher resolution by manipulating the chemical structure of the nucleotides in the AAUAAA sequence. In particular, since neither DNA nor 2'-O-methyl-containing RNA is polyadenylated (L. Conway,

FIG. 3. Analysis of complexes formed between AAUAAA specificity factors and 3'-truncated substrates. (A) Detection of complexes. Labeled RNAs were incubated in the DE-600 fraction (containing AAUAAA specificity factors) and analyzed by electrophoresis on nondenaturing polyacrylamide gels. Lanes: 1, -23/-5; 2, -23/-7; 3, -23/-9; 4, -23/+1 containing AAUACA instead of AAUAAA; 5, -18/-8 (AAUAAACCCC); 6, -18/-9 (AAUAAACCCC). Within each experiment, equal amounts of radioactivity were loaded on the gel for each sample. Naked RNA was run off the gel. Nonspecific complexes (i.e., complexes that form largely independently of RNA sequence) are not formed when the DE-600 fraction is used but are formed very rapidly in unfractionated nuclear extract (e.g., references 20 and 29). We infer that the components which form these complexes have been removed during fractionation. (B) Comparison of polyadenylation and complex formation. Efficiencies of polyadenylation (from Fig. 2C) and complex formation were quantitated by densitometry. Complex formation efficiency was averaged from three independent experiments for -23 RNAs and two experiments for -18 RNAs. The efficiencies are presented relative to the value for -23/-5 as 100% for the -23 RNAs and relative to the value of -18/-8 as 100% for the -18 RNAs. P, Polyadenylation; C, complex formation. -23/-9 and -18/-9 RNAs were not detectably polyadenylated.

A. Lamond, and M. Wickens, unpublished data), we first focused on the role, if any, of the 2' hydroxyl (2'-OH) groups present in the AAUAAA sequence. We tested the effect of removing the oxygen atom from the 2'-OH group of a single nucleotide, the U in AAUAAA. To do so, we prepared short substrates containing only a single U, that in AAUAAA. We generated substrates that differed only in the ribose moiety of this single nucleotide by transcription in vitro in the presence of either UTP or 2'-dUTP. The abilities of these two types of RNA to support polyadenylation were then compared.

Figure 4A shows a time course of polyadenylation in vitro for -20/-7 RNA containing either a normal ribose-containing uridine (rU) (lanes 1 to 3) or 2'-dU (lanes 4 to 6). The transcript containing 2'-dU was polyadenylated less efficiently.

To test whether this result was peculiar to the particular

FIG. 4. Demonstration that the 2'-OH of the U in AAUAAA is required for efficient polyadenylation. (A) Comparison of -20/-7 RNAs containing either rU or 2'-dU as the substrate for polyadenylation. -20/-7 single-U RNA (GGAAUAAACAAGAA) was incubated in the Mono Q fraction plus the DE-600 fraction at 30°C for the time (in minutes) indicated above each lane. -20/-7 single-U RNA containing 2'-dU (GGAAAdUAAACAAGAA) was incubated in the same fractions for identical times (lanes 4 to 6). (B) Comparison of -38/+1 RNAs containing either rU or 2'-dU as the substrate for polyadenylation. -38/+1 single-U RNA (GGCAACCACCACAAGCCGCAUAAACAAGCCAACAACA) was incubated in Mono Q plus DE-600 at 30°C for the time (in minutes) indicated above each lane. -38/+1 single-U RNA containing 2'-dU was incubated in the same fractions for identical times (lanes 5 to 8). (C) The fraction of RNAs containing rU and 2'-dU in the substrate and the product. -38/+1 single-U RNAs labeled with [α - 32 P]ATP were digested to completion with RNase T₂, and the digestion products were separated by two-dimensional thin-layer chromatography. Panels: 1, -38/+1 RNA; 2, -38/+1 RNA prepared by using 2'-dUTP in the transcription reaction; 3, the polyadenylated products generated with -38/+1 RNA, prepared by using 2'-dUTP, after incubation in Mono Q and DE-600 for 30 min; 4, the RNA that failed to react during this incubation. Polyadenylated and nonpolyadenylated reaction products were isolated from a polyacrylamide gel and shown to be purified away from one another by a second round of electrophoresis. As determined by densitometric scanning of the autoradiograms, the ratio of rUp to dUp in the starting RNA (panel 2) was 0.5:1, and the ratio in the polyadenylated RNA (panel 3) was 15:1. We corrected for the fact that 1 mol of dUpAp would contain twice the radioactivity of 1 mol of rUp, since the transcript was labeled with [α - 32 P]ATP. Using the formula fold preference = ratio of rUp to dUp in the product (15:1) divided by ratio of rUp to dUp in the starting RNA (1:2), we deduce that polyadenylation exhibits a 30-fold preference for rU over 2'-dU. This calculation requires that a twofold difference in substrate concentration result in a twofold difference in the amount of product generated, as is true under these conditions of enzyme excess (not shown). Differential stability of rU- and dU-containing RNAs, if it exists, does not affect the result, since the vast majority of the RNAs remained intact during the incubation.

RNA used, the same analysis was performed with a derivative of -38/+1 RNA that contains only a single U. This RNA contains the natural SV40 sequence except that the only U is the U in AAUAAA; all other U's have been changed to C. This RNA is polyadenylated as efficiently as natural, unmodified -38/+1 RNA (P. Wigley, unpublished data). Again, as

with $-20/-7$ RNAs, lack of the 2'-OH dramatically impaired polyadenylation (Fig. 4B).

The 2'-dUTP preparation used to generate these transcripts was contaminated with unmodified rUTP (J. Milligan and O. Uhlenbeck, personal communication; Fig. 4C). Therefore, the RNA generated in a transcription reaction using this dUTP preparation is a mixed population: some RNAs contain rU, whereas other RNAs contain 2'-dU. It was therefore essential to determine whether the polyadenylated products produced by using this mixed population contained rU, 2'-dU, or both. To quantitate the relative amounts of rU- and 2'-dU-containing RNAs, $-38/+1$ RNAs (labeled with [α - 32 P]ATP) were digested to completion with RNase T_2 , and the products were analyzed by two-dimensional thin-layer chromatography (Fig. 4C). RNase T_2 cleaves after each ribonucleotide, generating 3'-ribonucleotides. It does not cleave after 2'-deoxynucleotides. Thus, T_2 digestion of RNA containing a single U will generate rUp if the RNA contains rU but will instead generate 2'-dUpAp if the RNA contains 2'-dU. As a result, the ratio of rUp to 2'-dUpAp can be used to determine the relative abundance of the two types of RNA in a mixed population.

As expected, RNase T_2 digestion of RNA transcribed in the presence of rUTP yielded only unmodified rUp as well as Ap and Cp (Fig. 4C, panel 1; no Gp was detected because the RNA was labeled with [α - 32 P]ATP and contained no GpA dinucleotides). RNA transcribed in the presence of 2'-dUTP generated both rUp and dUpAp, as expected (Fig. 4C, panel 2). To assess unambiguously the relative ability of rU- and dU-containing RNAs to support polyadenylation, we determined the proportions of rU and dU in polyadenylated RNA produced by using this mixed population as a substrate. Almost all of the polyadenylated RNA contained rUp, not dUp (Fig. 4C, panel 3). In contrast, RNA that failed to react during the incubation (i.e., not polyadenylated) was enriched for dUp (panel 4). From the ratios of rUp to dUp in the substrate (0.5) and in the polyadenylated products (15.0), we deduce that polyadenylation exhibits a 30-fold preference for rU over 2'-dU (see legend to Fig. 4C for details).

The requirement for a 2'-OH group is not observed outside of AAUAAA. To investigate the specificity of the requirement for a 2'-OH group, 2'-dC was incorporated into $-20/-7$ RNA, which contains a unique C residue immediately 3' to AAUAAA. We compared the polyadenylation of this RNA (Fig. 5A, lanes 4 to 6) with that of the same RNA containing rC (lanes 1 to 3). These two RNAs were polyadenylated with the same efficiency. Moreover, RNase T_2 analysis demonstrated that the 2'-dC-containing RNA contained only deoxycytidine: dCpAp was generated, but rCp was not (Fig. 5B). Thus, we infer that removal of a 2'-OH group in the nucleotide immediately downstream of AAUAAA does not affect polyadenylation. In this sense, the requirement for a 2'-OH group is specific for the U in AAUAAA. Whether 2'-OH groups elsewhere in AAUAAA are also essential has not yet been determined.

The 2'-OH group is required for interaction with specificity factors. To determine how the lack of a 2'-OH group prevents polyadenylation, we analyzed the binding of specificity factor to 2'-dU-containing RNAs, using a gel retardation assay. Labeled $-38/+1$ RNA, containing either rU or 2'-dU in AAUAAA, was incubated with unfractionated extract (Fig. 6A, lanes 1 to 6) or with fractions of the extract that contain AAUAAA specificity factor but no detectable poly(A) polymerase (Fig. 6A, lanes 7 to 14). In both cases, the 2'-dU-containing RNA did not support the formation of

FIG. 5. Specificity of the 2'-OH requirement. (A) Comparison of $-20/-7$ RNAs containing either rC or 2'-dC as the substrate for polyadenylation. $-20/-7$ single-U RNA (GGAAUAAACAAGAA) was incubated in Mono Q plus DE-600 at 30°C for the time (in minutes) indicated above each lane. This RNA contains a single U and a single C and is designated C in the figure. $-20/-7$ single-U RNA containing 2'-dC (GGAAUAAAdCAAGAA) was incubated in the same fractions for identical times (lanes 4 to 6). Lanes 1 to 3 are identical to lanes 1 to 3 in Fig. 4A. (B) Demonstration that $-20/-7$ RNA transcribed with 2'-dCTP contains only 2'-dC. [α - 32 P]ATP-labeled $-20/-7$ single-U RNA, prepared by using 2'-dCTP, was digested with RNase T_2 , and the digestion products were separated by two-dimensional thin-layer chromatography. Positions of each monophosphate and of dCpAp are indicated. The position of the rCp spot, determined in parallel by using a different RNA, is indicated by the broken circle. No radioactivity was detected in this position.

polyadenylation-specific complexes as efficiently as did normal, rU-containing RNA. The specificity of the complexes formed on the rU- and 2'-dU-containing $-38/+1$ RNAs was demonstrated by comparison with a longer, $-58/+1$ RNA (Fig. 6A, lanes 15 to 18): the slowly migrating complexes formed on all $-38/+1$ and $-58/+1$ RNAs comigrated (Fig. 6A, lanes 15 to 17), and as demonstrated with the $-58/+1$ RNA, their formation required AAUAAA, since it was abolished by a mutation to AAUCAA (Fig. 6A, lane 18). The components in the extract that gave rise to nonspecific complexes were missing from the specificity factor fraction; as a result, RNA that failed to enter complexes after incubation with this fraction comigrated with free RNA (Fig. 6A, lanes 7 to 18). On the basis of these results, we conclude that the 2'-dU-containing RNA interacts inefficiently with components in the specificity factor fraction, presumably with specificity factor itself.

To confirm this conclusion and to quantitate the magnitude of the effect, we determined the ratio of rU and 2'-dU in those RNAs that formed complexes with specificity factor (Fig. 6B). [32 P]ATP-labeled 2'-dU-containing RNA was incubated with the specificity factor fraction for 2 min. Specific complexes were then purified by gel electrophoresis. RNA from these complexes was analyzed by RNase T_2 digestion and two-dimensional thin-layer chromatography (Fig. 6B, panel 1). As a control, we analyzed RNA that was incubated with specificity factors but failed to form a complex (Fig. 6B, panel 2).

The dU-containing substrate, before incubation with specificity factor, yielded both rUp and 2'-dUpAp, in a 1:2 ratio, as already shown (e.g., Fig. 4). In contrast, RNA that entered the specific complex yielded predominantly rUp; the ratio of rUp to 2'-dUpAp spots derived from this RNA was 7.5:1 (Fig. 6B, panel 1). Thus, RNA containing 2'-dUp was

FIG. 6. Demonstration that the 2'-OH group is required for interaction with specificity factor. (A) Gel retardation assays using RNAs containing either rU or dU in AAUAAA. [α - 32 P]ATP-labeled -38/+1 RNA containing either rU or 2'-dU was incubated either in crude extract (lanes 1 to 6) or in the DE-600 (specificity factor) fraction (lanes 7 to 18). The duration of each incubation is given above each lane. For the 0-min time points, RNA was added to the extract and the reaction was stopped by the addition of sodium dodecyl sulfate-proteinase K within 15 s. Lanes: 1 to 3, rU-containing RNA incubated in extract for either 0 (lane 1), 0.5 (lane 2), or 2 (lane 3) min; 4 to 6, 2'-dU-containing RNA incubated in extract for either 0 (lane 4), 0.5 (lane 5), or 2 (lane 6) min; 7, rU-containing RNA before any incubation; 8 to 10, rU-containing RNA incubated in the DE-600 fraction for 0 (lane 8), 0.5 (lane 9), or 2 (lane 10) min; 11, 2'-dU-containing RNA before any incubation; 12 to 14, 2'-dU-containing RNA incubated in the DE-600 fraction for 0 (lane 12), 0.5 (lane 13), or 2 (lane 14) min. In lanes 15 to 18, each RNA was incubated in the DE-600 fraction for 2 min. Lane 15, -38/+1 RNA containing rU; lane 16, -38/+1 RNA containing 2'-dU; lane 17, -58/+1 RNA containing AAUAAA; lane 18, -58/+1 RNA containing AAUCAA. (B) The fraction of RNAs containing rU and 2'-dU in the specific complex and in RNA that fails to enter the complex. [α - 32 P]ATP-labeled -38/+1 RNA transcribed in the presence of 2'-dUTP was incubated with the DE-600 fraction for 2 min. Complexes were then separated from RNA that did not enter complexes by electrophoresis through a nondenaturing gel, precisely as shown in lanes 7 to 18 of panel A. From this nondenaturing gel, we recovered RNA from the specific complex as well as RNA that failed to enter the complex (free RNA in panel A). The two RNAs were digested with RNase T₂, and the products were separated by two-dimensional thin-layer chromatography. Panels: 1, RNA from the specific complex; 2, RNA that failed to enter the complex. Positions of each 3'-monophosphate and of 2'-dUpAp are indicated; a broken circle indicates the position of the faint 2'-dUpAp spot present in RNA from the specific complex. As determined by densitometric scanning of the autoradiograms, the ratio of rUp to dUp in the RNA from the specific complex (panel 1) was 7.5:1, and the ratio in the RNA that failed to enter the complex (panel 2) was 0.5:1. For further details on quantitation, see text and legend to Fig. 4.

preferentially excluded from the complex. As expected, RNA that failed to enter the complex yielded the same 1:2 ratio (rUp to 2'-dUpAp) as did the substrate (Fig. 6B, panel 2). This RNA was not enriched for 2'-dUpAp because only a small fraction of the substrate entered the complex in the brief, 2-min incubation with specificity factor. Comparing the ratios of rUp to 2'-dUpAp in the substrate (0.5) and in the RNA from the specific complex (7.5), we conclude that specificity factors exhibit at least a 15-fold preference for rU-containing RNAs. Thus, this defect in the interaction with specificity factor is nearly sufficient, quantitatively, to account for the 30-fold defect in polyadenylation activity (Fig. 4).

DISCUSSION

Our results demonstrate that AAUAAA not only is necessary but is virtually sufficient for polyadenylation of pre-cleaved RNAs. Very short substrates (as few as 14 nucleotides) are polyadenylated efficiently as long as they contain AAUAAA. The shortest oligoribonucleotide we have used that supports polyadenylation, AAUAAACCCCA, contains only 11 nucleotides (Fig. 1). RNAs lacking a single nucleotide from one end or the other are not polyadenylated, demonstrating that operationally, this 11-nucleotide RNA is a minimal substrate *in vitro*. Thus, AAUAAACCCCA is sufficient to interact with the polyadenylation machinery,

which contains at least a poly(A) polymerase and a factor that recognizes AAUAAA, possibly an snRNP (2, 4, 5). The requirement for 11 nucleotides reflects the minimal length of RNA capable of interacting productively with both an AAUAAA recognition factor and poly(A) polymerase, since polymerase alone can efficiently add adenylate residues to a tetranucleotide substrate (24).

AAUAAACCCCA is the shortest RNA we have used that is capable of being detectably polyadenylated. However, optimal polyadenylation requires eight or more nucleotides downstream of AAUAAA (Fig. 2). As a result, polyadenylation of AAUAAACCCCA is less efficient than that of an RNA which extends three more nucleotides downstream. A computer-assisted analysis of 262 mRNA sequences reveals that the poly(A) site is within seven nucleotides of AAUAAA in fewer than 4% of those mRNAs (Wickens, in preparation). This is consistent with our results and suggests that aside from any constraints imposed by the cleavage reaction, efficient polyadenylation *in vivo* may also be optimal with eight or more nucleotides 3' to AAUAAA.

The sequence between AAUAAA and the 3' end appears to have little influence on polyadenylation, consistent with its lack of conservation (Wickens, in preparation). As the 3' end is brought progressively closer to AAUAAA (Fig. 2), substrates necessarily differ in sequence yet yield a systematic and progressive reduction in activity. Furthermore, the CCCC of AAUAAACCCCA, the minimal substrate, is not found in any natural mRNA yet supports polyadenylation (Fig. 2). No single base between AAUAAA and the polyadenylation site of SV40 late mRNA can be essential, since chemical modification of each base in this region does not reduce polyadenylation (6). On the basis of these several independent lines of evidence, we deduce that the requirement for five nucleotides beyond AAUAAA reflects a requirement for a minimum absolute distance, not a specific sequence. We infer that the polymerase must simultaneously contact specificity factor and the 3'-OH group and cannot do so on too short a tether (see below). Different sequences should vary in precisely how many nucleotides beyond AAUAAA are required, depending on their three-dimensional structure, yet each should exhibit a minimum length requirement. This is observed by using three slightly different sequences in Fig. 2. Further experiments, using more substrates in which the sequence between AAUAAA and the 3' end is varied systematically, will be needed to test this prediction conclusively.

RNAs with fewer than eight nucleotides 3' to AAUAAA interact efficiently with specificity factors yet are poor substrates for polyadenylation (Fig. 3). Thus, addition of poly(A), and not association with specificity factors, is the step affected by the distance between AAUAAA and the 3' end. Either the poly(A) polymerase interacts less stably with 3'-shortened substrates or the polymerase interacts stably but has reduced access to the 3' terminus of the RNA.

Not surprisingly, sequences outside of AAUAAA can influence the efficiency of the reaction. We anticipate that these nucleotides influence the structure of the substrate and alter either the effective distance between AAUAAA and the 3' end (see above) or the accessibility of AAUAAA. To assess the influence of sequences outside of AAUAAA, systematic structural analyses may be necessary. We therefore stress, at this point, two main conclusions: very short oligoribonucleotides support polyadenylation, and a minimal distance between AAUAAA and the 3' end is required.

Specificity factors can interact with AAUAAA in the absence of poly(A) polymerase. AAUAAA-specific com-

plexes are formed with factors present in a specificity factor fraction that contains no poly(A) polymerase activity (Fig. 3 and 6). Furthermore, these complexes are very similar in electrophoretic mobility to those formed in unfractionated nuclear extract by using longer, more conventional substrates (not shown). Although our data demonstrate that polymerase is not required for interaction with AAUAAA, it remains possible that in unfractionated extract and *in vivo*, polymerase and specificity factors preassemble into a complex before interaction with the substrate.

The 2'-OH group of the U in AAUAAA is required for efficient polyadenylation (Fig. 4) and for the binding of specificity factor (Fig. 6). These effects are specific, since removal of the 2'-OH of the C immediately 3' to AAUAAA has no effect on polyadenylation (Fig. 5). The failure of 2'-dU-containing RNAs to interact with specificity factor is nearly sufficient, quantitatively, to account for their defect in polyadenylation (Fig. 4 and 6). Further analysis will be required to determine whether the 2'-OH group of each ribose in AAUAAA is required.

The 2'-OH group of the U in AAUAAA could be necessary for interaction with specificity factor either because the hydroxyl is a contact point for that factor or because the hydroxyl is required to form a structure in AAUAAA that is recognized via other contact points. Removal of the single oxygen atom at the 2' position can result in rather dramatic effects on nucleotide conformation, affecting the pucker of the ribose and the rotational position of the base relative to the sugar (19). Such changes in structure could perturb interactions distant from the hydroxyl group. Although removal of a single 2'-OH group thus can have effects at a distance, we stress that the effect we observe is specific for the 2'-OH of the U in AAUAAA; removal of the 2'-OH of the first nucleotide downstream of AAUAAA has no effect on the reaction.

AAUAAA may be recognized either by a protein or by an snRNP. If an snRNA recognizes AAUAAA by base pairing, then the 2'-OH of the U must be required for that interaction. Thus, the simplest interpretation implied by the data is that at least one of the components that interacts with AAUAAA is a protein. Further analysis of the precise involvement of the 2'-OH group will require purified specificity factor and an understanding of the structure of AAUAAA in the RNAs with which it interacts. Attaining both of these goals may be facilitated by the use of very short oligoribonucleotide substrates of the type described in this report.

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